IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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Serial No.: 10/568,194
Filed: 2/10/2006
Title: "Conditioned Cell Culture medium, Method to Obtain the Same and Use of it for Maintainance, Proliferation and Differentiation of mammalian cells

DECLARATION

I declare:

1. I am an inventor of the subject matter that is described and claimed in the above captioned patent Application.

2. My colleagues and I have carried out experiments confirming that MMH-CM (Met Murine Hepatocyte-Conditioned Medium) described in the patent Application US 10/568,194 sustains self-renewal of stem/progenitors cells by demonstrating that cells grown in this culture medium maintain their stemness markers and differentiating ability.

3. Furthermore I declare:

Different assays have been carried out in our laboratory to confirm MMH-CM properties. These have been carried out with stem cells, historically defined by their ability to self-renew, as well as to generate daughter cells of any of the hematopoietic lineages.

The presence and the isolation of stem and progenitor cells are usually carried out using the expression of surface CD34 antigen as a positive marker (Berenson RJ et al J. Clin Inv 1988; 81:951-955).

In order to determine the effect(s) of soluble factors released by murine hepatocyte MMH on stem/progenitor cells differentiation, highly enriched human $CD34^+$ cells were used as a source of hematopoietic progenitor cells (the use of $CD34^+$ as stemness model has been recently reviewed in Furness and McNagny, Immunol. Res. 2006, 34: 13-32).

$CD34^+$ cells were isolated, from either cord blood or peripheral leukapheresis, and long-term cultivated for a period spanning from 21 to 108 days in MMH-conditioned medium (MMH-CM). Notably, we found that $CD34^+$ cells cultivated as long as four weeks in MMH-CM (CD34-CM cells) expressed the haematopoietic stem cell markers CD133 and CD34 in a relevant percentage (6% and 3%, respectively).

Thus, the biological properties (stemness) of CD34-CM cells were tested both *in vitro* and *in vivo*.

To determine whether CD34 cells cultivated in MMH-CM (CD34-CM) retained hematopoietic activity, we performed colony-forming cell methylcellulose assay throughout the culturing period. As shown in Table 1 CD34-CM gave rise to hematopoietic colony-forming cell (CFC) up to after 28 days of culture in MMH-CM. They formed both CFU-GM and CFU-GEMM colonies. At day 21, a significant number of large colonies with poorly differentiated cells was observed.

This suggests that after the first three weeks in MMH-CM the absolute number of clonogenic cells increased with respect to the input cell population. Notably, cells from large colonies, originated from CD34-CM at day 21, when removed from the methylcellulose, dispersed and replated in fresh methylcellulose medium, gave rise to secondary colonies demonstrat-

ing, therefore, that they had maintained their high proliferative potential CFC.

4. Furthermore I declare:

The hematopoietic potential of the CD34-CM was tested up to 108 days cultivation in MMH-CM. After 108 days CD34-CM cells maintained the ability to generate colonies of the hematopoietic lineages.

Notably, we studied the hematopoietic activity potential of CD34-CM also *in vivo* using the NOD/SCID mouse model (Lewis ID et al. Blood. 1998; 91:630-640).

Cells cultured for 21 days in MMH-CM were transplanted in sublethally irradiated NOD/SCID mice. CD34-CM cell transplantation reduced mice mortality to a significant extent: 5/12 (41%) mice died in comparison to 17/18 (95%) in non transplanted mice, thus indicating that CD34-CM cells sustain hematopoiesis also *in vivo*.

All together these data indicate that growth in the MMH-CM sustain the hematopoietic potential of $CD34^+$ cells at long term (108 days) both *in vitro* and *in vivo*, therefore demonstrating its capability to induce the self renewal of progenitor/stem cells.

5. Furthermore I declare:

We found that when $CD34^+$ cells were cultivated as a bulk population they expressed also endothelial-specific markers and genes, as revealed by FACS and microarray analysis, respectively.

In order to test whether CD34-CM could differentiate also towards endothelial lineage, as in their potential, we performed both *in vitro* and *in vivo* endothelial functional assays.

We analyzed whether CD34-CM (cultured for 21 days) participated in the organization of vascular structures *in vitro* and we found that CD34-CM were able to grow in Matrigel and form vascular-like structures. Moreover, in mixed-culture with HMEC, CD34-CM were able to incorporate into newly forming vascular structures.

In vivo we found that CD34-CM cells cultured for 21 days, differentiated in CD144+ cells when transplanted into sub-lethally irradiated NOD/SCID mice, (about 1-2%, 68 weeks after transplantation). Moreover, in order to investigate the ability of CD34-CM to incorporate into newly forming vasculature, we utilized a VEGF-expressing glioblastoma tumor cell line, known for its high efficiency to promote angiogenesis.

CD34-CM, were infected with a lentiviral vector expressing GFP and were injected in nude mice, either intravenously after tumor implantation or subcutaneously together with tumor cells. The presence of CD34-CM in tumors was then evaluated both for GFP expression and through labeling with anti-human CD144 mAb (figure 1, panels (a) and (b)). CD34-CM cells were found in the tumor independently from the administration routes and the majority of them was incorporated or adhered to the tumor vasculature (figure 1, panel (c)).

Therefore even after a long term culture in MMH-CM, the $CD34^+$ stem cell

population retains both the hematopoietic and the endothelial potentials.

6. Furthermore I declare:

To further investigate whether these two differentiation programs arise from common or independent cellular precursors, we cultured single $CD34^+$ cell in MMH-CM and tested their expression of hematopoietic and/or endothelial markers. Using three different donors, 1800 $CD34^+$ cells were plated at clonal dilution (i.e. 1 cell/well). After 60 days we recovered 767 clones (43% clonal efficiency) formed by a limited and variable number of cells, spanning from 10 to 50-100 cells (figure 2, panel A-B). Some of these clones contained cells expressing both endothelial and hematopoietic markers (CD144 and CD45, respectively; figure 2 panel C). Clones kept in culture, for as long as 120 days, displayed neither significant cellular proliferation nor major morphological changes, highlighting the capability of MMH-CM to control their proliferative potential. Notably, clones kept in MMH-CM for 60 days, when dispersed and transferred to methylcellulose, gave rise to several secondary colonies (figure 2 panel D) indicating that CD34-CM retain clonogenic activity and that single $CD34^+$ cells in MMH-CM give rise to *bona fide* hemangioblasts, as characterized, for example, in Pelosi E et al. Blood 2002;100:3203-3208.

We concluded that the two different precursor originated from the same stem cell and thus that MMH-CM sustained the maintenance of both potentialities.

Overall these data confirm that *in vitro* MMH-CM is able to sustain in short and long term cultivation the self-renewal ability of human stem/progenitor cells.

75. Furthermore I declare:

MMH cell lines are known in the art and easily available to the skilled man by standard isolation techniques from liver of c-Met transgenic mice, available, since publication of their preparation in Amicone et al. The EMBO Journal, 1997; 16: 495-503) upon request to: Prof. Marco Tripodi, Fondazione Istituto Pasteur Cenci-Bolognetti, Dipartimento di Biotecnologie cellulari ed Ematologia, Università La Sapienza Roma, v. le Regina Elena 324, 00161 ROMA - ITALY.

8. All statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

Date: 06/10/08 Signature: Marco Tripodi

Printed name: MARCO TRIPODI

Enclosure A: Table 1

Table 1. Clonogenic potential of freshly isolated CD34+ and CD34-CM.

Cell fraction	Days of culture	CFCs	CFU-GEMM	CFU-GM
CD34+	0	144	109	35
CD34-CM	14	606	152	465
CD34-CM	21	230121	10120	129101
CD34-CM	28	4911	169	334

Human colony-forming cells (CFCs) were assayed in methylcellulose by standard methods. Data represent numbers of colonies obtained culturing 1000 cells of: $CD34^+$=$CD34^+$ cells freshly isolated from UCB (Umbilical Cord Blood); CD34-CM=highly enriched CD34+ cells cultured in MMH-CM for the indicated times. The results of 3 independent experiments are shown, each experiment was performed in triplicate. Abbreviations: CFU-GEMM, colony-forming unit granulocyte-erytrocyte-macrophage-megakaryocyte; CFU-GM, colony-forming unit granulocyte- macrophage.

Enclosure B:

- Figure 1
- Figure 2

Figure 1

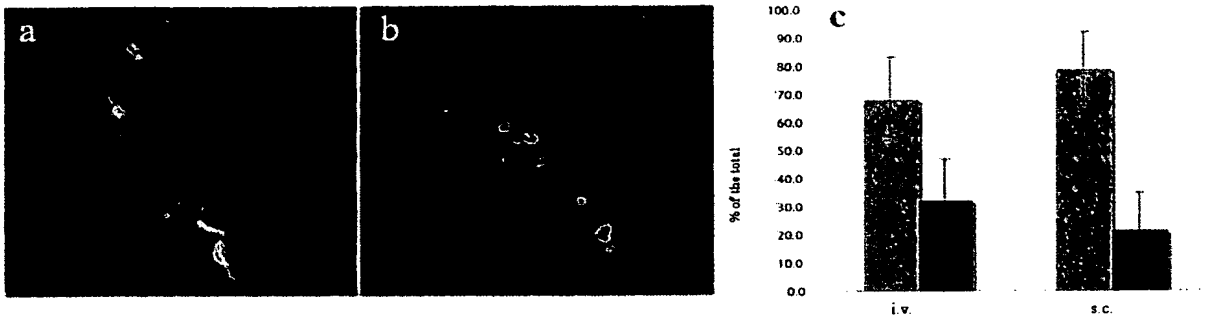

Figure 1. Incorporation of CD34-CM-GFP cells in newly forming vessels in C6 glioblastoma tumors. CD34-CM-GFP cells were injected either (a) i.v. 4 days after tumor implantation or (b) mixed with C6 glioblastoma cells before injection in mice. CD34-CM-GFP cells specifically associated with mouse microvessels. For each way of CD34-CM-GFP administration two independent experiments were performed, using at least three mice each. Two representative specimens stained with anti-mouse CD31 mAb (red staining) while human GFP cells (green labelling) are reported. Magnification 63X. (c) Percentages of CD34-CM-GFP cells associated (violet columns) or not associated (red columns) to tumor vasculature in experiments described in (a) or (b), indicated as i.v. or s.c., respectively. Data are expressed as mean±SD of cell counts from three mice. For each mouse at least ten tumor sections have been evaluated.

Figure 2

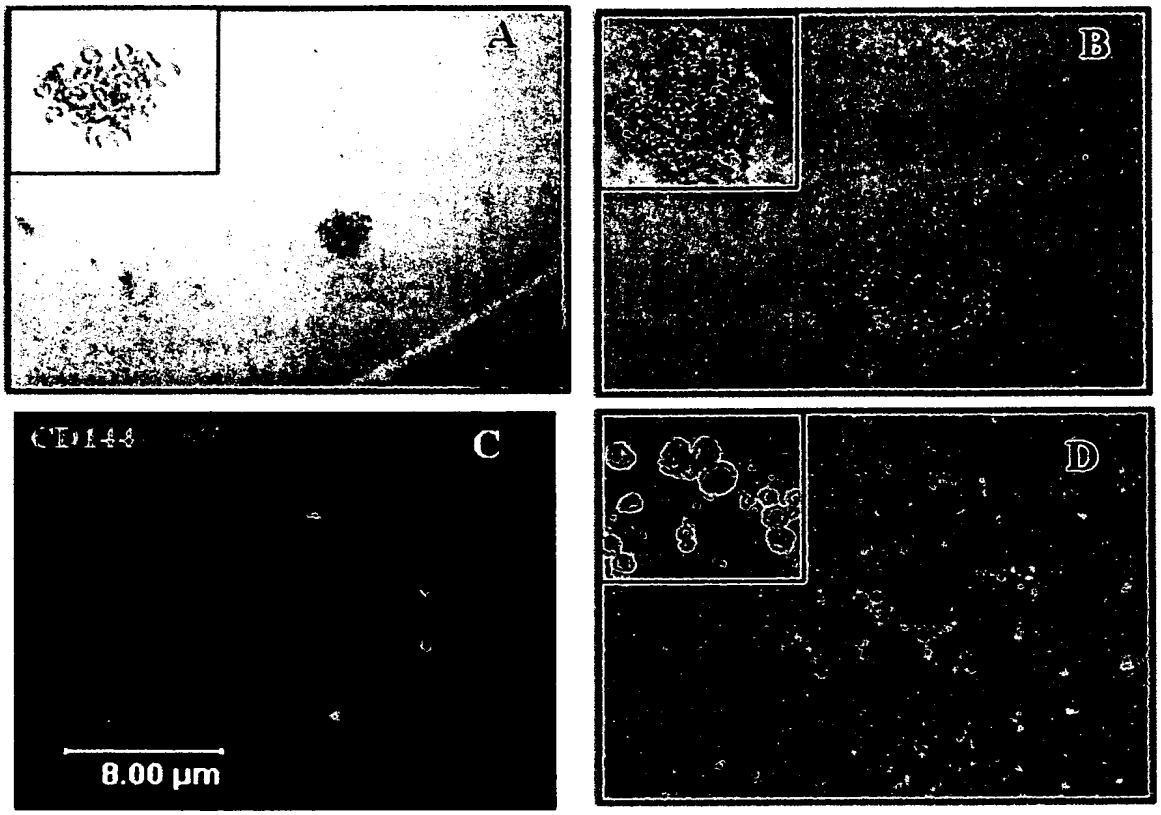

Figure 2. Characterization of clones originated from single CD34+ cells cultivated in MMH-CM. (A-B) Single CD34+ cells cultivated in MMH-CM for 60 days (single CD34-CM) formed clones of different size. Phase contrast micrographs, original magnification 20X. Inlets represent higher magnification of clones. (C) Single CD34-CM cells were double stained for CD144 (green) and CD45 (red) and assayed by confocal microscopy. Original magnification 63x. (D) Single CD34-CM clones at day60 of culture were moved to methylcellulose and observed after 2 weeks. Original magnification 20X.

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Beyond Mere Markers

Functions for CD34 Family of Sialomucins in Hematopoiesis

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Abstract

CD34, podocalyxin, and endoglycan are members of a family of single-pass transmembrane proteins that show distinct expression on early hematopoietic precursors and vascular-associated tissue. In spite of the fact that the expression of CD34 on these early progenitors has been known for over 20 yr and used clinically in hematopoietic stem cell transplantation for more than 15 yr, little is known about its exact role or function. More recently, CD34 expression has been shown to distinguish activated early progenitors from quiescent cells. With the subsequent identification of podocalyxin and endoglycan as related family members also expressed on early progenitor cells, attention is slowly shifting toward understanding how these molecules might contribute to progenitor function and behavior. In this review we examine the existing evidence and propose testable models to reveal the importance of these molecules for stem and progenitor cell function.

Key Words

CD34
Podocalyxin
Endoglycan
Sialomucin
Hematopoiesis
Hematopoietic stem cells
Adhesion
Migration
NHERF-1
NHERF-2

Introduction

CD34, podocalyxin and endoglycan are grouped as a protein family (henceforth termed the CD34 family) based on conservation of domain organization (Figs. 1A, C, and 2)*(1–3)* and their conserved genomic organization (Fig. 1A) *(2–4)*. At the genomic level all members are encoded by eight exons, with paralogous exons encoding paralogous protein domains, which is the strongest evidence for shared descent (Fig. 1A) *(2–4)*. This is important in establishing this group as a family because they do not naturally form a clade based solely on amino acid sequence similarity (Fig. 1B). To date, alternatively spliced variants of CD34 and podocalyxin have been identified, both of which make use of an alternative eighth exon resulting in mRNAs encoding proteins with a truncated cytoplasmic domain *(4,5)*. The structures of the three family members, based on biochemical and sequence information, consist of an extracellular N-terminal mucin domain followed by a

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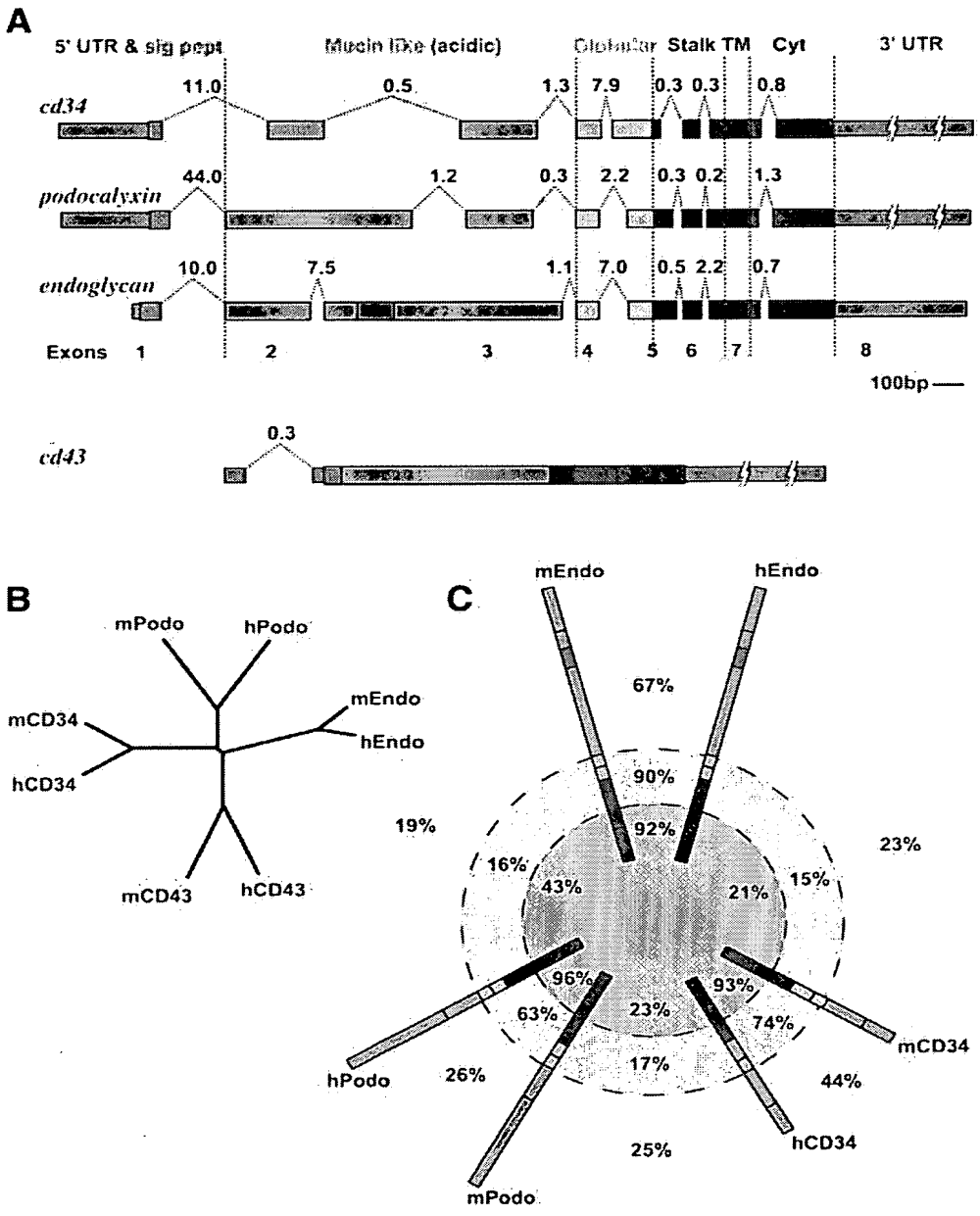


globular domain and stalk, with a single-pass transmembrane region and a charged cytoplasmic tail (Fig. 2). CD34 family members have been identified in a variety of vertebrates, including mouse, human, dog, and chicken and gene predictions have been made for zebrafish. No homologous proteins have been identified in urochordates, invertebrates, or

Fig. 1. Genomic structure and relationship based on amino acid sequence of CD34 family members. **(A)** Schematic representation of the genomic structure of human *CD34, podocalyxin, endoglycan,* and *CD43* (used as an outgroup) based on sequence contigs and genomic mapping available in the NCBI human sequence database. Numbers above dashed lines indicate intron size in kilobases, exon numbers are indicated below (and marked). The relative sizes of exons are shown to scale (see scale bar), with the exception of the 3′ UTRs of *CD34, podocalyxin,* and *CD43.* The location of coding sequences is indicated: signal peptide (sig pept–red), mucin-like domain (orange), globular domain (yellow), stalk (green), transmembrane (TM–cyan), and cytoplasmic tail (Cyt–dark blue). **(B)** An unrooted phylogram showing the relationship of human and mouse CD34, podocalyxin, endoglycan, and CD43 (used as an outgroup) proteins indicating the difficulty in assigning CD34, podocalyxin, and endoglycan to a single family based solely on amino acid sequence. Branch lengths are proportional to calculated distance. **(C)** A schematic representation of the relationship of CD34, podocalyxin, and endoglycan deduced amino acid sequences highlighting the sequence conservation differences between the various domains and family members. Color scheme follows that of **A,** numbers indicate % identity between neighboring proteins, and domains have been grouped: mucin like, globular + stalk domain, transmembrane + cytoplasmic tail. Human (h) and mouse (m) proteins are given for comparison.

other eukaryotes and thus it seems likely that the parental gene arose among the first vertebrates, followed closely by gene duplication. These proteins, therefore, appear to have arisen to accommodate some aspect of vertebrate body plan not shared by other chordates.

Mucin domains are common to a wide range of extracellular and transmembrane proteins including the mucins, sialomucins (to which the CD34 family belongs), lectin ligands, as well as some transmembrane receptors. Mucin domains are heavily modified by O-linked carbohydrate moieties and are usually coded by one exon only. They are characterized as being rich in serine, threonine, and proline and are generally poorly conserved at the primary amino acid level. In the case of the CD34 family, the mucin domains are sparsely modified with N-linked and heavily modified with O-linked glycosylation *(1,6,7).* Much of the O-linked glycosylation is decorated with sialic acid and this, combined with sulfation *(8–10),* contributes to a high net negative charge. The mucin domain of CD34 family members is capable of binding the leukocyte homing receptor, L-selectin *(10–12)* (Table 2). However, this interaction is highly dependent on a particular pattern of glycosylation *(9,10)* that has only been observed in specialized lymph node endothelial cells known as high endothelial venules (HEV) *(13–15).* To date, despite their conservation (Fig. 1C), no specific function has been attributed to either the globular or stalk domains of any family member. The cytoplasmic tail is presumed to have important functions, based on its high sequence conservation and has been shown to be subject to signal-regulated phosphorylation *(16–18)* and to bind several intracellular ligands (Table 1, and see below) *(19–25).* The transmembrane region and cytoplasmic tail are the most conserved motifs between family members and are extremely well conserved orthologously (Fig. 1C).

CD34

CD34 Identification and Expression

CD34 was first identified as an antigen expressed on hematopoietic progenitors in screens of monoclonal antibodies generated against human hematopoietic precursors *(26–28).* The subsequent demonstration that the CD34 positive (CD34+) cell fraction of bone marrow could successfully engraft both baboons *(29)* and humans *(30)* has led to it becoming one of the most widely used markers of hematopoietic stem cells, with well

Table 1. Tissue Distribution of CD34 Family Memebers

Tissue/cells	CD34	Podocalyxin	Endoglycan
Multipotent hematopoietic progenitors			
Adult	+	+	+
Embryonic	+	+	?
Prescursors			
Erythroid	–	+	?
Thrombocytic	+	+	?
Myeloid	+	–	?
Lymphoid	+	–	?
Mature Hematopoietic cells			
B cells	–	–	?
T cells	–	–	?
Macrophages	–	–	?
Granulocytes	–	–	?
Eosionphils	–	–	?
Mast cells	+*	–	?
Erythrocytes	–	+†	?
Platelets	–	+	?
Vascular endothelia	+	+	+
Smooth muscle	–	–	+
Podocytes	–	+	?
Brain(neural)	–	+¥	+
Mesothelial boundary elements	–	+	?

* Murine mast cells only.

† Embryonic and anemic erythrocytes only.

¥ Ependymal layer only.

over 12,000 Medline citations. In spite of the vast amount of literature on CD34 as a marker, there is only limited and conflicting information regarding its function.

CD34 Endothelial Expression

Beyond its expression on HSCs (see below), CD34 is expressed on vascular endothelia in the adult *(31–35)*. During development, too, CD34 is on all vascular endothelia with highest expression in small vessels and filopodia of angiogenic sprouts *(35,36)*. This expression includes those endothelial cells surrounding the blood islands of the yolk sac *(35–37)* as well as endothelia of the aorta–gonad–mesonephros (AGM) region *(36,38,39)*. These CD34+ endothelial and stromal cells from the AGM have been used, in vitro, for long-term expansion of HSC populations *(39,40)*. In the adult, high expression has been reported on the endothelia of vessels in all organs, primarily in small vessels *(31,33,41,42)*.

CD34 Expression on Stromal and Mesenchymal Stem Cells

Mesenchymal stem cells are nonhematopoietic cells that reside in the bone marrow and are capable of giving rise to various cells involved in the maintenance of bone and bone marrow, including osteoblasts, chondrocytes, adipocytes and stromal cells. CD34 has been reported to be expressed on a subset of stromal cells of bone marrow origin *(43–45)*, a subset of muscle-derived progenitor cells that are distinct form satellite cells *(46)*, as well as placental stromal cells *(47)*. Both of

these bone marrow– and muscle-derived progenitor cell populations appear to represent an early progenitor of stromal cells that may be functionally equivalent to the proposed mesenchymal stem cell (MSC). There is broad evidence supporting the model that the CD34+ subset of stromal cells might represent a type of MSC. In vitro, these cells have high fibroblast colony forming unit potential (CFU-F) *(45)* and, when isolated from muscle, are capable of contributing to regeneration of damaged muscle and bone *(46)*. Most importantly, human CD34+ stromal cells are capable of reconstituting stroma of sublethally irradiated NOD/SCID mice *(48)* suggesting that the CD34+ population does contain MSCs. However, there are also reports that both human *(49)* and mouse *(44)* mesenchymal stem cells are not CD34+. In the case of Hung et al. *(49)* the absence of CD34 may be an effect of long-term culture, prior to analysis, or may be a reflection that there is more than one type of MSC. Peister et al. *(44)* analyzed CD34 expression on MSCs from a variety of inbred mouse strains after long-term culture and found expression in two mouse strains and lack of expression in two others. Again, it is unclear whether this real difference between strains, in terms of endogenous MSCs, is an effect of long-term culture or is due to epitope differences between mouse strains (insufficient information is provided about the antibody used to make a judgement in this regard). The balance of evidence supports the idea that an early multipotential stromal precursor exists and this precursor can be CD34+.

CD34 Expression in the Hematopoietic System

The first evidence of CD34 as a marker of hematopoietic stem/progenitor cells came from FACS analysis demonstrating that its expression is restricted to a subset of bone marrow cells and absent from terminally differentiated hematopoietic lineages *(26,28)*. Moreover, sorting of CD34+ cells greatly enriched for colony forming units (CFUs) of a variety of lineages *(26–28)*. The subsequent demonstration that the CD34+ fraction of bone marrow can fully reconstitute the hematopoietic system of baboons and humans *(29,30)* confirmed that these cells had properties of HSCs. Owing to the clinical utility, in terms of improved hematopoietic engraftment, achieved using sorted CD34+ human bone marrow or mobilized progenitor donor cells, CD34 became widely accepted as a marker of HSCs *(50)*. The fact that CD34+ cells from the bone marrow and mobilized peripheral blood display HSC activity does not exclude the possibility that CD34– cells also display this activity. In fact a number of groups have reported that CD34– cells are capable of long-term reconstitution of the hematopoietic system *(51–56)*. Hematopoietic reconstitution by CD34– stem cells was demonstrated first in mice *(51–53)* and subsequently in primates, including humans, using a xenotransplantation model *(54–56)*. However, definition of these HSCs as CD34– relies on absence of staining with monoclonal antibodies whose epitope recognition is unchanged by neuraminidase and glycoprotease cleavage *(36,57)* (implying recognition of the globular or stalk region) to analyse cell surface expression. Since Jones et al. *(51)* reported very low levels of CD34 mRNA in CD34– HSCs, it is formally possible that CD34 is expressed on these cells but the epitope is unavailable for recognition by the antibodies, or CD34 expression is being posttranslationally regulated, for example, by being maintained in intracellular stores. Independent studies in mice, using the same type of antibody, demonstrate that HSCs are present in both CD34+ and negative cell popula-

tions *(58–60)*. At the time of publication, these reports were controversial because CD34 expression had been widely used as a key clinical method for separating HSCs for transplantation and as a prognostic indicator of reconstitution efficiency *(61)*. Some resolution has been forthcoming with in vivo data from mice providing evidence that CD34 expression is reversible and dependent on stem cell activation status *(62)*. Further to this, it has been shown for both human *(63)* (in a xenotransplantation model) and mouse *(64)* that CD34 expression on HSCs is reversible and that both CD34– and CD34+ stem cells are competent for serial transplantation and reconstitution.

Whether a particular stem cell expresses CD34 or not may correspond to the activation status of the cell *(65–67)*. Quiescent HSCs are CD34–, whereas G-CSF mobilized cells are CD34+ *(65)*. Quiescent HSCs are largely in G_0 and granulocyte colony-stimulating factor (G-CSF) mobilized stem cells have been also reported to be in G_0/G_1 *(68,69)*. This suggests that CD34 expression is not strictly cell cycle dependent and does not require stem cell division, merely activation. Activation and cell cycle regulated expression in HSCs may reflect an aspect of CD34 regulation that is true also for stromal precursors and may, thus, resolve the anomalies between reports regarding its expression in this cell type. This raises the possibility that CD34 may play an important role on a variety of activated precursors.

This recent evidence, that CD34 expression on HSCs is related to activation status, dovetails well with the observations concerning hematopoietic CD34 expression during development. The earliest sites of hematopoiesis during development are in the blood islands of the yolk sac *(70)* and the AGM region, which is the first site of definitive hematopoiesis *(71)*. In both regions, hematopoietic progenitors show CD34 expression *(34–36,72)*. Shortly after the development of the liver primordia, the liver is colonized by hematopoietic progenitors and for the rest of embryogenesis this becomes the principal site for definitive hematopoiesis *(73)*. Again, hematopoietic progenitors in the fetal liver express CD34 *(34,35,71,74,75)*. In all these developmental sites for hematopoiesis there is massive expansion of the cell population as well as repeated mobilization in order to seed new compartments. The expression of CD34 in these developmental compartments provides further support for the idea that CD34 may have some important function on activated or mobilized HSCs.

It is important, having discussed CD34 expression on HSCs, to note that CD34 appears not to be expressed on any mature hematopoietic cells with the exception of murine mast cells *(76)*. Although the current evidence suggests that expression on *mature* mast cells is a feature specific to the murine hematopoietic system *(77–80)*, in both humans and mice the data suggests committed mast cell *precursors* express CD34 *(76,77,81)*. Bone marrow mast cell progenitors are somewhat elusive because they appear morphologically and phenotypically indistinguishable from CD34+ HSCs *(76,81)* and are also present at the same frequency as HSCs *(82)*. The similarity between mast cell progenitors and HSCs may also present the converse problem, that various types of analyses in which HSC cell surface phenotype is used could be confounded by the presence of mast cell precursors.

Although CD34 does appear to be absent from most mature hematopoietic lineages, it is expressed on a subset of bone marrow multipotent precursors of the thrombocytic, myeloid, and a very small fraction of thymic precursors *(83,84)*. Because these precursors are difficult to identify, either by morphology or by cell surface antigens, the assessment that these are in

fact precursors of particular lineages depends on performing colony assays, or limiting dilution reconstitution assays. This leaves open the question of how early a precursor is really marked by the presence of CD34. In any case, the fact that these precursors express CD34, while it is not expressed by mature lineages, hints that this molecule may be important for a precursor function, not present in terminally differentiated hematopoietic cells.

CD34 Function

In light of the above discussion on CD34 expression, particularly that it is associated with HSC activation and mobilization, the obvious question is what function does this protein serve? CD34 has been proposed to function as (1) an inhibitor of differentiation and promoter of proliferation in HSCs, (2) an adhesion molecule, possibly capable of signaling, and (3) an anti-adhesion molecule. Thus far, the only ligand or counterreceptor identified for CD34 is L-selectin and the ability of these two molecules to interact is exquisitely dependent on a particular glycosylation pattern on CD34, only occurring in the specialized cells of HEVs. Here we will evaluate the evidence for each functional model.

CD34 and Proliferation/Differentiation

As discussed above, CD34 expression is limited to non-quiescent or activated hematopoietic precursors and is absent from differentiated hematopoietic lineages. This correlates with the idea that it may have roles in inhibition of differentiation and promotion of proliferation. The main confirmatory evidence that CD34 might play a role in inhibition of differentiation comes from overexpression studies in a hematopoietic progenitor cell line. Fackler et al. *(85),* showed that overexpression of full-length CD34, but not the naturally occurring form with a truncated cytoplasmic domain, inhibits in vitro differentiation of the myelomonocytic M1 cell line. They interpreted this as evidence that the cytoplasmic tail of CD34 provides signals that inhibit differentiation. Because the overexpression construct used resulted in protein levels far beyond endogenous, it is difficult to say whether this reflects a normal function of CD34. It is possible, for example, that this overexpression may have resulted in either constitutive signaling or squelching of an alternative pathway by a molecule that may or may not normally signal during hematopoietic differentiation.

The only other data linking CD34 to maturation and proliferation focused on a strain of CD34– null mice. In these studies CD34– null embryonic stem (ES) cells and cells derived from CD34– null yolk sac, fetal liver, adult bone marrow, and spleen exhibited lower CFU activity in vitro, when compared with wild-type–derived counterparts *(86).* In ES cells, this phenotype can be rescued by transfection of full-length CD34, or the naturally occurring splice variant encoding CD34 with a truncated cytoplasmic domain *(86).* The simplest interpretation here is that CD34 is required, at normal expression levels, for natural proliferation or differentiation and maturation of progenitor cells. However, in the light of subsequent evidence that CD34 is a regulator of adhesion, it is possible that these maturation defects are actually downstream of an adhesion defect (see below).

CD34 as a Pro-Adhesive Molecule

There is one line of evidence supporting a direct adhesive role for CD34; it has been shown that CD34, when expressed on HEVs, is suitably modified for binding L-selectin *(11)* and thus serves a pro-adhesive role in leukocyte homing. On HEVs a number of glycoproteins are reported to be modified with a HEV-specific glycosylation (sulfated sialyl-

Lewis x *(13)*) and this modification serves as a specific ligand for L-selectin and is required for leukocyte homing (8). CD34 is one of at least four glycoproteins that carries this unusual HEV-specific modification *(11,87)*, is thus capable of supporting L-selectin–dependent lymphocyte rolling in vitro *(88)* and is responsible for up to 50% of L-selectin–dependent tethering *(88)*. In spite of this, in vivo, L-selectin–dependent lymphocyte rolling is unaffected in CD34– null mice *(89)* and lymphocyte homing appears normal *(89)*. CD34– null mice do, however, show a defect in allergen-induced eosinophil recruitment to the lung *(89)*, a defect not reported in an independent CD34 knockout strain *(86)*. It seems likely there is functional compensation by other L-selectin ligands on HEVs, and this accounts for the discrepancy between the in vitro and in vivo data.

CD34 and Signaling

There is some evidence for signal-regulated phosphorylation of CD34, with Fackler et al. *(17)* showing that PKC could serine phosphorylate CD34 resulting in upregulated surface expression *(18)*. The site of PKC phosphorylation has not been identified; however, there are seven conserved serines in the cytoplasmic tail of CD34; six of which give high scores for predicted phosphorylation, but only one is a predicted PKC site *(90,91)*. It would be useful to have this site formally demonstrated because phosphorylation is a common modification for regulation of protein–protein interactions. It has also been shown that CD34 is subject to tyrosine phosphorylation *(92)*, although the kinase responsible is unknown. CD34 is thus subject to a number of regulatory post-translation modifications, suggesting that its function might be tightly controlled. They also hint that CD34 might be a signaling molecule. In the absence of a known ligand for CD34, as it is post-translationally modified on most cells, several groups have evaluated potential outside-in signaling capacity of CD34 by crosslinking with antibodies. A variety of anti-CD34 monoclonal antibodies exist and have been classified according to their epitope sensitivity to neuraminidase and glycopeptidase *(93)*. Those antibodies sensitive to either treatment recognize the mucin-like domain of CD34, whereas those that are resistant to both recognize the globular, or stalk, domain. Crosslinking using antibodies of all classes leads to clustering of CD34 *(94)* in hematopoietic precursors; however, only antibodies recognizing the mucin domain can induce capping and tyrosine kinase activation *(94)*. CD34 capping, induced by crosslinking with mucin-directed antibodies, results in actin polymerization and tyrosine phosphorylation of both Lyn and Syk membrane-associated tyrosine kinases *(94)*. Similarly, it is antibodies of this class that are capable of inducing both homotypic *(95,96)* and heterotypic *(95,97)* adhesion of hematopoietic cells. These data support the idea that CD34 has a role in blocking adhesion when uniformly distributed. The antibody-induced capping of CD34 and subsequent homotypic adhesion are ATP, calcium, and tyrosine kinase dependent, and are at least partially mediated by activation of β2 integrin and ICAM-1 *(95,96)*. These data argue that CD34 is actively involved in cell signaling cascades that regulate cell–cell adhesion and that CD34 cell surface expression and localization are regulated by intracellular signals. The limitations of the data relating to CD34's ability to induce intracellular signaling cascades are that neither the intracellular mediators of this signaling nor a ligand or counterreceptor have been identified. Thus, there is no information on the mechanism that may shed light on a function for CD34. The intracellular SH2 family adaptor CrkL has been identified as a potential interactor with CD34 *(19)* (Table 1), however,

there is not yet any information about the conditions under which this interaction might occur and whether this adaptor is responsible for signal transduction through CD34.

CD34 as an Antiadhesive Molecule

Recently we showed that, in mouse, CD34 is a selective marker of mast cells *(76)*. We have subsequently exploited this observation, and the ability to culture pure populations of primary mast cells, as a means to address CD34 function *(98)*. Bone marrow mast cells (BMMC) from CD34 knockout mice display higher homotypic adhesion compared with mast cells from wild-type animals, a phenotype that can be reversed by transfection of full-length or naturally occurring truncated CD34 *(98)*. The version with a truncated cytoplasmic domain appears to function as a more potent inhibitor of adhesion than the full-length molecule *(98)*. This supports a model of CD34 being an antiadhesion molecule and suggests that the cytoplasmic tail is important to allow proper regulation of this antiadhesive property. When used in competitive reconstitution assays, CD34– null cells show a significant defect in their ability to contribute to the peritoneal mast cell population and to reconstitute bone marrow when compared with wild-type cells *(98)*. Thus, it seems that the antiadhesive role of CD34 might be important in the ability of mobilized precursors to migrate to the bone marrow or to find the appropriate niche. In light of CD34's ability to block adhesion and yet show a crosslinking-dependent ability to induce adhesion, we speculate that CD34 may passively block adhesion via its bulky, negatively charged mucin domain, and yet actively signal this change in adhesion behavior when clustered or crosslinked (Fig. 3A).

CD34 So Far

How far have we come in understanding CD34's role in the hematopoietic system? We now know that CD34 definitively marks a subset of hematopoietic progenitors and that these are non-quiescent cells involved in migration or proliferation. So far the gene deletion studies have provided little illumination, with two independent knockouts published with different but mild hematopoietic defects and no apparent defect in other tissues expressing CD34 *(86,89)*. This argues for redundancy in function, which may be provided by distantly related proteins, such as CD43, or the other CD34 family members, podocalyxin and endoglycan (see below). Interpretation of the relatively small amount of molecular data has, until recently, been hampered by the absence of any clear indication of function either from primary amino acid sequence; genetic evidence, or identification of a ligand or counterreceptor. The accumulating evidence suggests that CD34 is actively involved in regulating adhesion by serving as an antiadhesion molecule *(98)*. CD34 can be uniformly distributed over the cell surface and block adhesion through stearic hindrance, via the bulky mucin domain and possibly by signaling to inhibit the activity of adhesion molecules (Fig. 3A). Under alternative conditions, either via indirect signaling or possibly by CD34 binding to a ligand or counterreceptor, active relocalization of CD34 occurs and reveals adhesion molecules (Fig. 3A). CD34 may then be involved in inside-out signaling to activate adhesion molecules and signaling cascades. Thus, we propose that CD34 has an important role in facilitating migration of various hematopoietic progenitors to their appropriate niche, for example, mast cells to the peritoneum *(98)* or eosinophils to the lung *(89)*.

Podocalyxin

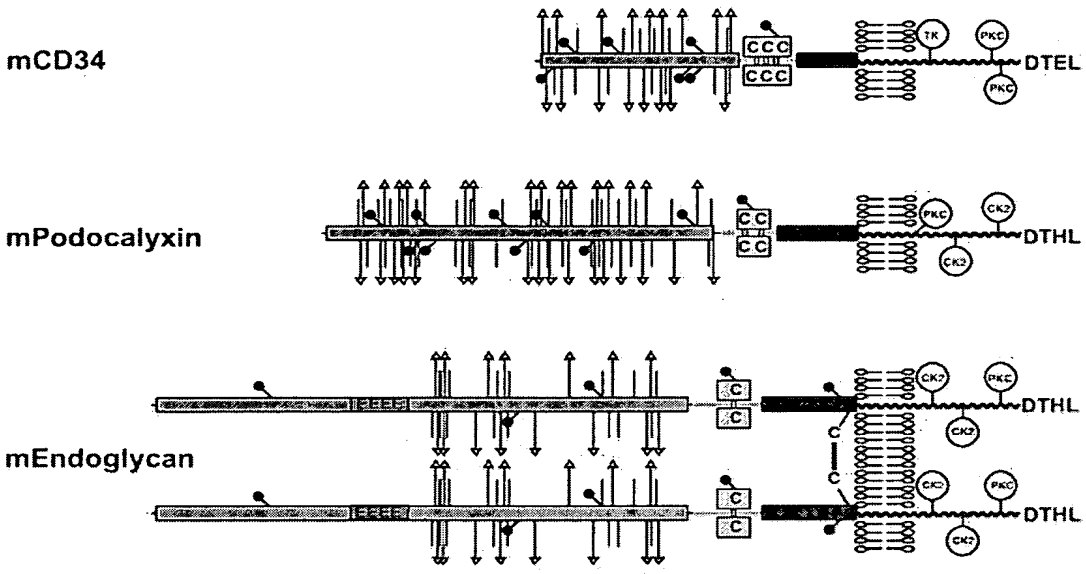
Podocalyxin Identification and Expression

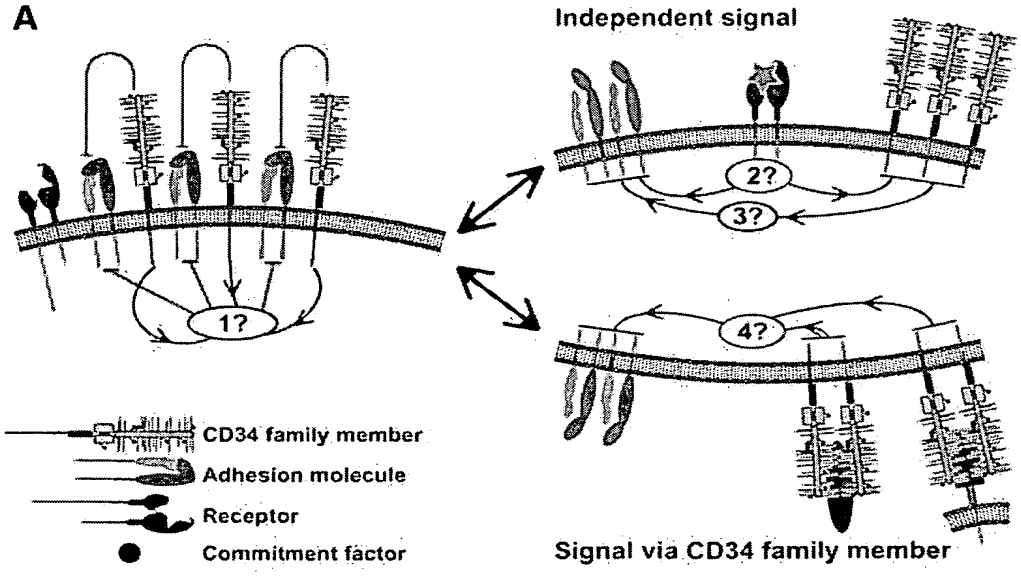
Podocalyxin [also known as Myb-Ets-transformed progenitor (MEP) 21, podoca-

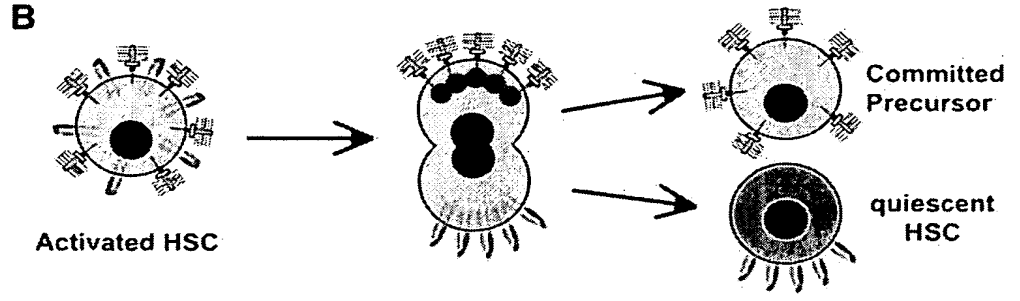
Fig. 2. Schematic of primary protein structure. Scheme follows that of Fig. 1A [from left-hand side (N-terminus) mucin–globular-stalk–transmembrane–cytoplasmic tail] with the poly acidic stretch of endoglycan indicated in purple. Angled lines with filled circles represent potential NH_2-linked carbohydrates, horizontal bars with and without arrows indicate potential O-linked glycosylations, arrows are potential sialic acid modified O-linked glycosylations. Cysteines available for disulfide bond formation are indicated by C, consensus phosphorylation sites for Tyrosine Kinase (TK), Protein Kinase C (PKC), and Casein Kinase 2 (CK2) are indicated as is the C-terminal PDZ docking motif (DTE/HL).

lyxin-like protein 1 (PCLP1), thrombomucin, and gp135] was first identified as a marker of kidney glomerular podocytes *(6)*. To date the most detailed analysis of its function and activity has been conducted in kidney cells. The most noticeable differences between podocalyxin's and CD34's structures are that podocalyxin's small globular domain contains two, instead of three, paired cysteines, and a much larger mucin-like domain comprising roughly 250 amino acids compared with about 120 in CD34. In terms of non-hematopoietic expression, podocalyxin is expressed on the primitive endoderm, ectoderm, and the first intraembryonic mesodermal precursors *(99)*. It is also expressed by hemangioblasts of the AGM *(100,101)* and throughout development on the luminal face of vascular endothelia *(100,102)*, on the kidney glomerular podocytes *(6,100,103)*, neuroepithelium in the primitive ectoderm *(99)*, and subsequently throughout boundary elements in the brain *(104)*. It is also on other boundary elements such as the mesothelial linings and luminal faces of newly formed cavities *(99,100)*. In the adult, Podocalyxin is expressed on kidney podocytes *(6,102)* and all vascular endothelia *(102)*. It will be apparent to the reader that the embryonic endothelial expression appears to mimic that of CD34, although it has not been shown that podocalyxin positive (podocalyxin+) cells from the AGM are the same cells as those expressing CD34, or that these podocalyxin+ cells possess mesenchymal/stromal stem cell activity. It is tempting to think that these family members might be capable of functional compensation in these tissues (see Table 1 for a comparative overview of expression). Likewise, CD34 and podocalyxin show coincident vascular endothelial expression in the adult, and podocalyxin has been demonstrated to be modified appropriately on HEVs to enable binding of L-selectin *(12)*.

Fig. 3 Proposed models for family function. **(A)** Cartoon of the surface of a cell in non-adhesive mode is shown on the left, in which a CD34 family member prevents adhesion by stearic hindrance via the mucin-like ectodomain while also blocking inside out signaling (1?) responsible for adhesion molecule activation. On the right is shown two alternative mechanisms by which CD34 family members might be involved in upregulating cell adhesion. In the top cartoon a ligand binds a cell-surface receptor, such as a cytokine receptor, triggers intracellular signaling which results in partitioning of CD34 family members (2?) away from cell adhesion molecules. This leads either, to signaling from the receptor (2?), or from clustered CD34 family members (3?) activates adhesion molecules, as represented by extended structure. In the bottom cartoon either a soluble ligand (purple) or counter receptor (light teal) binds CD34 family member that transduces a signal (4?) necessary for partitioning of, and activation of adhesion molecules. **(B)** Cartoon showing how CD34 family members might be involved in asymmetric cell division. As a hematopoietic precursor divides the CD34 family member localizes to one pole of the dividing cell and drags with it factors involved in precursor commitment (shown in purple) (such as β-catenin) allowing maintenance of pluripotential by that daughter cell whilst also allowing the other daughter cell to differentiate.


mCD34
DTEL
mPodocalyxin
DTHL
mEndoglycan
DTHL
DTHL


A
Independent signal
2?
3?
1?
4?
CD34 family member
Adhesion molecule
Receptor
Commitment factor
Signal via CD34 family member


B
Committed
Precursor
quiescent
HSC
Activated HSC

Podocalyxin Expression in the Hematopoietic System

The first description of podocalyxin on hematopoietic cells came from studies in chickens, with the identification of MEP21/thrombomucin as a marker of normal and transformed multipotent hematopoietic progenitors *(100,105,106)*. Subsequently, podocalyxin expression has been reported on hematopoietic precursors in mice *(99,101)* and humans *(107)* and this expression is maintained on early hematopoietic cells throughout development *(99)*. As with CD34+ HSCs, those expressing podocalyxin are capable of long-term reconstitution in mice *(99)*. It has not yet been reported whether CD34 and podocalyxin are coexpressed on these HSCs, nor whether the expression of podocalyxin on these cells is activation/proliferation dependent, as is the case with CD34. This expression profile and reconstitution capacity supports the notion that these family members could provide functional compensation in the hematopoietic system.

Podocalyxin is also expressed beyond hematopoietic progenitors and is present on thrombocytes in chicken *(100)* and the functionally equivalent cells (platelets) in rats *(108)* as well as their precursors (megakaryocytes) *(108)*. In resting platelets much of the podocalyxin is present in intracellular pools and it is significantly upregulated at the cell surface in response to stimulation with thrombin *(108)*. This seems to mirror data regarding CD34 on HSCs, showing that it is mobilized to the cell surface in response to signaling *(18,66)*. Podocalyxin is also expressed on nucleated erythroid cells and this expression correlates closely with high rates of erythropoiesis, including anemic responses, and seeding of erythroid progenitors to new hematopoietic microenvironments *(99)*.

Podocalyxin Function and Comparison with CD34

In the kidney glomerulus, Podocalyxin is expressed on the surface of specialized structures called podocyte foot processes *(6)*. Podocytes develop from epithelial cells that face the lumen of the glomerular capsule and their foot processes form an interdigitating network of cell extensions, with narrow filtration slits (slit diaphragms) that allow the passage of filtrate into the capsule. Podocalyxin is the major sialoprotein of these foot processes *(6,102)* and systemic treatment with agents that remove or neutralize the surface negative charge from the foot processes results in collapse of these structures (effacement) leading to kidney dysfunction *(6,109–111)*. That podocyte foot processes are dependent on podocalyxin is unequivocal, because, in podocalyxin-null animals, these structures fail to form and the podocyte epithelial precursors retain tight junctions, resulting in anuria and perinatal mortality *(2)*. The widely accepted model for how the mature foot process structures are maintained by podocalyxin expression is that podocalyxin's high net negative charge maintains the slit diaphragms via charge repulsion. This is consistent with the reported data, however it has not been demonstrated that the treatments that neutralize or remove negative charge from podocalyxin do not also affect its localization. Moreover, podocalyxin is not detected in the filtration slits, but rather on the more apical luminal face of the foot processes *(6)*. It is also possible that the decoration of the mucin domain with this high net negative charge is important for some aspect of signaling via podocalyxin to maintain these structures. This is supported by the observation that treatments resulting in the removal of this negative charge result in uncoupling of podocalyxin from the cytoskeleton *(24)*. In podocyte

foot processes, podocalyxin is bound at its very C-terminus to the adaptor protein NHERF-2 (Na(+)/H(+) exchange regulatory factor 2, also known as E3KARP [Na(+)/H(+) exchanger (NHE) type 3 kinase A regulatory protein] and SLC9A3R2 (Solute carrier family 9 isoform A3 regulatory factor 2) *(20,23,24)*. NHERF-2 is a cytosolic scaffolding and signaling protein containing two tandem PDZ (PSD-95, Dlg, ZO-1) domains and a carboxyl terminal ERM (ezrin, radixin, moesin) binding domain. NHERF-2 has been shown to interact with a number of transmembrane proteins via one or other of its PDZ domains [for review see Volz et al. *(112)*]. It can link these proteins to the cytoskeleton via its own interaction with ERM family members, which then interact with the actin cytoskeleton *(113)*. Interaction of the ERM family members with both the actin cytoskeleton and their binding partners is signal-regulated *(113)*. The NHERF family of proteins have complex and incompletely understood roles in regulation of membrane protein trafficking, activity, and recruitment of signaling molecules *(112)*. In podocytes, podocalyxin, NHERF-2, and ezrin form a complex that presumably is capable of linking podocalyxin to the actin cytoskeleton *(24)*. NHERF-2 shows extremely restricted expression, and we do not find it expressed in any hematopoietic tissues (unpublished observation), although its close relative NHERF-1 is *(25)*. Given the similarity between NHERF-1 and -2, it has been assumed that both these proteins would bind podocalyxin. NHERF-1 and podocalyxin have been shown to colocalize in the canine kidney cell line MDCK *(21,22)* and both a recombinant fusion protein containing podocalyxin's cytoplasmic tail *(22)* and transiently transfected tagged NHERF-1 *(24)* can co-immunoprecipitate each other. In addition, we have shown that, in hematopoietic cells, there is an interaction of endogenous proteins *(25)*. The interaction between podocalyxin and NHERF-1 occurs via NHERF-1's second PDZ domain and the C-terminal 4 amino acids (DTHL) of podocalyxin *(24,25)*. This C-terminus has the alternate sequence DTEL in CD34 and, in vitro, although podocalyxin's tail interacts with NHERF-1, CD34's tail does not *(25)*. Moreover, we are able to immunoprecipitate only a very small fraction of NHERF-1, with anti-podocalyxin antibodies, from early hematopoietic precursors, suggesting that although the in vitro interaction is very stable, the cellular interaction is either transient or regulated dynamically by a signal we have yet to identify *(25)*. Podocalyxin also appears to be a target for phosphorylation (as has been shown for CD34) in response to phorbol esters, although we have yet to identify the kinase responsible (unpublished data). Overexpression of podocalyxin in MDCK cells leads to activation of RhoA (Ras homolog A) *(22)* and enhanced phosphorylation of NHERF-1 *(22)*. Schmieder et al. *(22)* argue that this is due to an interaction between podocalyxin and ezrin (via NHERF-1) displacing RhoGDI (Ras homolog Guanine nucleotide Dissociation Inhibitors) and thus activating RhoA *(22)*. However, because RhoA signaling can operate upstream or downstream of ezrin [for review see Ivetic and Ridley *(114)*], it is possible that podocalyxin overexpression results in a signaling cascade where ezrin activation is the consequence of RhoA activation rather than vice versa. In either case it is clear that forced overexpression of podocalyxin allows it to direct, or participate in, signaling events.

CD34 does not appear to associate with NHERF-1 (Table 2), suggesting that if functional compensation exists between these two family members in HSCs, NHERF-1 does not participate in the shared pathway. By the same analogy, the reported binding site for CrkL on CD34 is completely absent from podoca-

Table 2. CD34 Family Member Ligands

Ligand	Family members bound	Cell type	Type of interaction
Extracellular			
L-selectin	**CD34, podocalyxin, endoglycan**	**HEV**	**Sialyl lewis-x carbohydrate dependent** (refs. *10–12*)
Intracellular			
NRERF-1	**Podocalyxin, endoglycan**	**Hematopoietic cells** **Breast cancer**	**C-terminal PDZ interaction (DTHL)** (refs. *22,24,25*)
NHERF-2	**Podocalyxin**	**Podocyte**	**C-terminal PDZ interaction (DTHL)** (refs. *20,21,23*)
ERM	**Podocalyxin**	**MDCK**	**Juxtamembrane (HQRISQRKDQQR)** (ref. *22*)
CrkL	**CD34**	**Hematopoietic cells**	**Juxtamembrane** (RRSWSPTGER*) (ref. *19*)

Amino acid sequences of interaction motifs are given, with critical residues in bold. *In the case of CD34–CrkL the critical residues within the sequence habe not been mapped. References (see main text) are given.

lyxin's cytoplasmic tail, implying that CrkL is not participating in a shared hematopoietic pathway.

Podocalyxin So Far

Podocalyxin was first identified as the major sialoprotein of the glomerular foot processes and it is clear that it has an important role in maintaining these structures. Experiments conducted so far do not address the mechanism by which abrogation of podocalyxin's negative charge results in collapse of these structures. The model in which charge repulsion alone maintains the filtration slits does not fully address the subtleties involved in maintenance of these structures. First, it appears that podocalyxin is localized in a manner inconsistent with maintenance of slits by charge repulsion alone, being restricted to the apical domain of the foot processes, which faces the lumen of the glomerulus. Second, effacement of the foot processes involves loss of adhesion of these structures to the basal lamina rather than the formation of tight junctions seen in podocalyxin-null animals. Furthermore, podocalyxin's localization after treatment to abrogate the negative charge has never been assessed, nor has the effect of this treatment on signaling to various junctional complexes.

In terms of hematopoiesis, it is clear that podocalyxin expression marks a subset of HSCs, but further research needs to be conducted to analyze whether this represents the same, distinct, or overlapping subsets compared with CD34. The genetic evidence for podocalyxin function in hematopoiesis is difficult to interpret, because null mice are not viable. Delineation of podocalyxin function would thus benefit from a conditional knockout. As for CD34, the functional data from gene targeting and overexpression of podocalyxin support the notion that podocalyxin is involved in signal-regulated loss of cell adhesion (see Fig. 3A).

Endoglycan

Endoglycan Identification, Expression, and Function

Endoglycan is the most recently identified member of the CD34 family *(1)*. The most notable difference from CD34 and podocalyxin is endoglycan's mucin domain, which is predicted to be approx 350 amino acids and also contains a poly glutamic acid stretch of almost 30 uninterrupted residues. In addition, endoglycan has only a single pair of cysteines in the globular domain and an unpaired cysteine in the juxtamembrane region, which is presumed to allow homodimerization (Fig. 2). Endoglycan mRNA is highly expressed in the brain, pancreas, and kidney with very low expression in the liver *(1)*. Preliminary data suggest expression on vascular smooth muscle *(1)*. All hematopoietic tissues have low levels of mRNA with highest expression in the lymph nodes *(1)*. FACS analysis also shows expression of endoglycan on the CD34+ fraction of bone marrow. Beyond its expression on CD34+ cells from the bone marrow and on HEVs, nothing is known of the exact cell types that normally express endoglycan. Based on staining data it is assumed (but not shown) that endoglycan, like podocalyxin and CD34, is expressed on a subset of hematopoietic precursors and endothelia. As with the other members of the CD34 family, endoglycan can function as a ligand for L-selectin *(10,115)*.

CD34 Family

We are starting to see some of the important roles that the CD34 family of proteins have in various aspects of hematopoiesis. Presently, we are at a point where the field needs some thorough molecular analysis and side-by-side comparison of the expression of the three members. It will be important to establish under what conditions early progenitors express these proteins and whether their expression is regulated in a coordinate manner, as would be necessary for any functional compensation. A summary of what is presently known about their expression is given in table 1. Promoter analysis would be another avenue of research that may provide clues to the roles of these proteins, especially because the expression of CD34 (at least) is upregulated as HSCs are activated/start to proliferate. Basic information about the nature of these proteins in signaling should be established. All members have consensus phosphorylation sites for (at least) CK2 (Casein Kinase 2) and PKC and, based on CD34, are probably also targets for phosphorylation by tyrosine kinases (TK). It would be valuable to establish which kinases are capable of signal-regulated phosphorylation. Although sequence analysis would indicate that endoglycan is capable of covalent homodimerization, analysis of the function of all members would benefit greatly from data regarding oligomerization. One of the current models for regulation of adhesion by CD34 family members is that they act as an umbrella to stearically hinder adhesion molecule function; inactive integrins extend approx 23 nm above the lipid bilayer and, based on heights of classical mucins, CD34, podocalyxin, and endoglycan would be expected to project 33, 55, and 77 nm, respectively. Experimental confirmation of this would add weight to this model but would still not address what signaling mechanisms, normally associated with integrin function, are activated in response to capping of CD34 family members. Lastly the function of the globular and stalk domains needs to be addressed.

Obviously we have put forward a strong case for the CD34 family playing an active role as antiadhesion molecules in regulating

cell–cell and cell–substrate adhesion (see Fig. 3A). The expression of both CD34 and podocalyxin (and probably endoglycan) are restricted to subsets of hematopoietic precursors, and both are capable of being localized to one pole of these cells. Therefore, there may be instances in which CD34 family members partition to one daughter cell. With the identification of NHERF-1 as a ligand for podocalyxin there are even more intriguing possibilities. NHERF-1 (and a number of other PDZ-containing proteins) interacts with β-catenin and so, via NHERF-1 (or alternative PDZ protein), CD34 family members may have a role in asymmetric cell division by dragging important transcription factors, such as β-catenin, to only one daughter cell (see Fig. 3C).

Now is an exciting time for this family of proteins, the pressure generated due to CD34's use as a pan-HSC marker has abated. With this encumbrance gone, research focus can now shift to understanding the essence of what these proteins actually do!

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Antigen CD34$^+$ Marrow Cells Engraft Lethally Irradiated Baboons

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Abstract

The CD34 antigen is present on 1–4% of human marrow cells including virtually all hematopoietic progenitors detected by in vitro assays. Since the anti–CD34 monoclonal antibody 12-8 reacts with a similar marrow population in baboons, it was possible to test whether this antigen is expressed by stem cells responsible for hematopoietic reconstitution in vivo. CD34$^+$ cells were enriched from marrows of five baboons using avidin-biotin immunoadsorption. After lethal irradiation, the five animals were given 15–27 $\times$ 10^6 autologous marrow cells (3.2–4.4 $\times$ 10^6 cells/kg) containing 65–91% CD34$^+$ cells. All animals achieved granulocyte counts > 1,000/mm^3 and platelet counts > 20 $\times$ 10^3/mm^3 by 13–24 d posttransplant and subsequently developed normal peripheral blood counts. Two additional animals received 184 and 285 $\times$ 10^6 marrow cells/kg depleted of CD34$^+$ cells. One animal died at day 29 without engraftment, while the other had pancytopenia for > 100 d posttransplant. The data suggest that stem cells responsible for hematopoietic reconstitution are CD34$^+$.

Introduction

Studies of human hematopoietic stem cells have been limited by the inability to identify and isolate these progenitor cells from marrow. Monoclonal antibody technology has made it possible to characterize cell surface antigens expressed by human hematopoietic progenitors and their progeny (1, 2). The CD34 antigen identified by antibodies 12-8, MY-10, BI-3C5, and ICH3 is of particular interest because it is primarily found on immature hematopoietic elements in human marrow (3–6). Nearly all colony-forming progenitors, granulocyte-macrophage colony-forming units (CFU-GM)[1], erythroid burst-forming units (BFU-E), and multipotential colony-forming units (CFU-MIX), detectable with in vitro assays express the CD34 antigen. Antibody 12-8 also identifies the precursors of colony-forming cells in long-term marrow culture (5). In nonhuman primates, this antibody also reacts with 1–4% of marrow cells that contain virtually all hematopoietic colony-forming progenitors (7). To test whether CD34$^+$ marrow cells were capable of restoring hematopoiesis in vivo, lethally irradiated baboons were transplanted with autologous CD34$^+$ marrow cells enriched by avidin-biotin immunoadsorption chromatography (8–11).

Methods

Baboons (Papio cynocephalus) and animal treatment

Baboons that were born at the University of Washington Regional Primate Center were placed in isolation and dewormed prior to use. The animals weighed 3.9–5.4 kg and ranged 1–2 yr in age. Research was conducted at the Primate Center under conditions that met National Institutes of Health standards as stated in the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. NIH 85-23, 1985), Institute of Laboratory Animal Resources recommendations, and American Association for Accreditation of Laboratory Animal Care accreditation standards for animals of this species. A central venous catheter was placed in all animals. Marrow was obtained by aspiration of both femora and processed using previously established procedures (12). After marrow aspiration, all animals were given 9.2 Gy of total body irradiation as a single exposure delivered from two opposing ^{60}Co sources at a rate of 7 cGy/min.

Peripheral blood counts were determined daily until normal counts were achieved and then weekly thereafter. Serum electrolytes as well as other laboratory tests and X-rays were performed as clinically indicated. Parenteral fluid and electrolytes were given based on clinical status (e.g., vomiting and diarrhea) and laboratory values. Prophylactic antibiotics (piperacillin 100 mg/kg t.i.d. and gentamicin 1.7 mg/kg t.i.d.) were administered intravenously starting immediately posttransplant and continued until animals achieved a granulocyte count > 500/mm^3. The animals were given whole blood transfusions from random baboons for treatment of severe and life-threatening anemia and thrombocytopenia. All blood products used for transfusion were irradiated in vitro (15 Gy). Gross and histologic postmortem examinations were carried out on all animals that died.

Antibodies and immunofluorescence staining reagents

Antibody 12-8 (murine IgM isotype) was partially purified from ascites fluid using boric acid precipitation (13). Affinity-purified goat anti-mouse IgM antiserum was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL) and biotinylated as previously described (9). The fluorescein isothiocyanate conjugate of avidin (avidin-FITC) was obtained from Vector Laboratories (Burlingame, CA).

Production of avidin-Biogel

Avidin obtained from Calbiochem-Behring Corp. (San Diego, CA) was conjugated to Biogel P-30 (Bio-Rad Laboratories, Richmond, CA) using a minor modification of a previously published procedure (9, 10). To make the gel used for positive cell selection, carboxylated Biogel P-30 was treated successively with 10 μg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC-HCl) and 100 μg of avidin per ml of gel. To make the gel used for depletion, Biogel P-30 was treated successively with 20 μg/ml EDC-HCl and 1 mg of avidin for each ml of gel.

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1. *Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; CFU-GM, granulocyte-macrophage colony-forming unit; CFU-MIX, multipotential colony-forming unit.

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Coupling of avidin to the gel was nearly quantitative under these conditions so that each milliliter of gel used for positive selection contained 100 μg of avidin and each ml of gel used for depletion contained 1 mg of avidin.

Antibody treatment, column separation, and FACS sorting of column-separated cells

Antibody treatment. Nucleated marrow cells (50×10^6/ml) obtained from a buffy coat preparation were incubated with antibody 12-8 (50 μg/ml) in phosphate-buffered saline (PBS) with 1% bovine serum albumin (PBS/BSA) for 30 min at 4°C. The cells were washed twice and then incubated at the same cell concentration in PBS/BSA with 1 μg/ml of biotinylated goat anti-mouse IgM antiserum for an additional 30 min at 4°C. The cells were again washed twice and adjusted to a concentration of $75–100 \times 10^6$ cells/ml in PBS/BSA for column treatment.

Isolation of $CD34^+$ cells. The antibody-treated cells were passed over a Chromaflex 15 × 2.5-cm column (Kontes Co., Vineland, NJ) containing 20 ml avidin-Biogel (100 μg/ml avidin) at a flow rate of 6–12 ml/min until a total volume of ~ 100 ml had been collected. ~ 50 ml of PBS was then passed through the gel at the same flow rate to wash out BSA. The adherent cells were dislodged by mechanical agitation with a 10-ml pipette until a total volume of 100 ml had been collected. The recovered adherent cells were directly infused in three animals.

In two animals, recovered adherent cells were further separated by FACS prior to infusion. In this procedure, the adherent cells at a concentration of 10^7 cells/ml were incubated with a 1:100 dilution of avidin-FITC in PBS/BSA for 30 min at 4°C. The cells were washed twice and then separated by flow microfluorimetric sorting on a FACS IV (Becton Dickinson Immunocytometry Systems, Mountain View, CA) as previously described (5). The cells with a level of fluorescence intensity > 98% of unlabeled cells were collected and infused into these two animals.

Depletion of $CD34^+$ cells. Nucleated marrow cells were treated successively with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum as described above. These cells at a concentration of 75×10^6 cells/ml were passed over a Chromaflex 25 × 2.5-cm column containing 100 ml of avidin-Biogel (1 mg/ml avidin) at a flow rate of 1 ml/min. The nonadherent cells that washed through the column were collected and infused.

Immunofluorescence studies

The percentage of cells reactive with antibody 12-8 was determined by immunofluorescence staining and flow microfluorimetric analysis by using an indirect immunofluorescence procedure (9, 10). Briefly, 5×10^5 cells were incubated with a 1:100 dilution of avidin-FITC in PBS/BSA with 0.1% sodium azide for 20 min at 4°C. The stained cells were washed before and after exposure to hemolytic buffer and then analyzed with a FACS IV and computer 440 (Becton Dickinson Immunocytometry Systems). The unseparated and adsorbed cells labeled with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum were stained with avidin-FITC. As a negative control, the unlabeled marrow cells were stained with avidin-FITC or incubated successively with a nonreactive, control IgM antibody and biotinylated goat anti-mouse IgM antiserum and stained with avidin-FITC. The percentage of $CD34^+$ cells was determined by subtracting the percentage of cells positively staining that were labeled with the control reagents from the percentage of cells positively staining after labeling with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum.

Colony-forming cell assays

Cells from unseparated and separated baboon marrow were cultured at 10^4 to 10^5 cells per 35-mm culture dish in semisolid agar culture medium. Medium consisted of Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY) supplemented with 20% fetal bovine serum (Hyclone, Logan, UT), 3 IU/ml human urinary erythropoietin (Terry Fox Cancer Center, Vancouver, BC, Canada), 10% human placental conditioned medium, 10^{-4} M 2-mercaptoethanol, and 0.3% (wt/vol) agar (Seapaque, FMC Corp, Rockland, ME). Medium conditioned with human placental tissue was prepared as a crude source of colony-stimulating activities by the method of Schlunk and Schleyer (14). Cultures were incubated at 37°C, 5% CO_2 in a humidified incubator and scored for different colony types after 14–16 d using an inverted microscope.

Results

Transplantation of enriched $CD34^+$ cells. Using antibody 12-8, cells expressing the CD34 antigen were positively selected from the marrows ($0.8–3.6 \times 10^9$ cells) of five baboons (Table I). In three experiments, $20–27 \times 10^6$ cells adherent to the column were recovered. These cells were 65–81% $CD34^+$ and represented 1.6–2.5% of the starting cell number applied to the column. Fig. 1 shows an example of the enrichment of $CD34^+$ cells in the column adherent population compared with the unseparated marrow cells from one of the baboons. In two additional experiments, recovered column-adherent cells were separated by flow microfluorimetric sorting to further enrich $CD34^+$ cells. This yielded 19 and 15×10^6 cells that were 85 and 91% $CD34^+$ and accounted for 0.5% and 0.6% of the starting number of marrow cells, respectively. The committed hematopoietic progenitors including CFU-GM, BFU-E, and CFU-MIX were enriched in the positively selected cell populations (Table II). The $CD34^-$ cells isolated by flow microfluorimetric sorting from the column-adherent population contained few colony-forming cells.

After 9.2 Gy of total body irradiation, the five animals received $3.2–4.4 \times 10^6$ selected cells/kg ($2.7–3.5 \times 10^6$ $CD34^+$ cells/kg). All animals achieved granulocyte counts > 1,000/mm³ and platelet counts > 20×10^3/mm³ by 13–24 d, and received their last whole-blood transfusions 8–21 d posttransplant (Table I). Marrow aspirates and biopsies obtained 3–4 wk after transplantation showed normal marrow cellularity and the presence of all hematopoietic lineages in all animals. Fig. 2 shows that the temporal pattern of recovery of leukocytes and platelets in these baboons was similar to that observed in control animals given unprocessed marrow (see below).

Transplantation of marrow depleted of $CD34^+$ cells. Two animals received 0.9 and 1.2×10^9 cells (184 and 285×10^6 cells/kg, respectively) depleted of $CD34^+$ cells by immunoadsorption with antibody 12-8 (Table I). This treatment resulted in a 1–2-log reduction in detectable colony-forming cells (Table II). One animal died at day 29 without evidence of engraftment. The second animal demonstrated marrow aplasia for > 2 mo after transplantation. This animal had a granulocyte count < 100, platelet count < 20,000, and continued to require transfusions for > 100 d posttransplant. This baboon continued to have severe myeloid hypoplasia documented on serial marrow examinations until its death on day 227.

Control animals. Three animals received 206, 218, and 270 $\times 10^6$ cells/kg of unprocessed marrow after total body irradiation. Two animals engrafted with a temporal pattern of hematologic recovery similar to that of the animals transplanted with $CD34^+$ cells (Table I and Fig. 2). The third animal developed severe gastrointestinal bleeding and died at day 19 posttransplant with a total white blood cell count of 1,500, a granulocyte count of 172 (day 15), a platelet count of 7,000/mm³,

Table I. Baboon Marrow Transplants: Cell Separation and Engraftment Data

	Baboon	Original cells	Separation procedure	Cells infused			Day posttransplant			Survival
				Total		$CD34^+$	Neutrophils	Platelets	Last transfusion	
		$\times 10^6$		$\times 10^6$	$\times 10^6/kg$	%	>1,000/mm³	>20,000/mm³		d
Enriched	1	1,500	Column	27	4.1	65	13	14	13	103
	2	1,500	Column	24	4.4	81	20	18	17	110
	3	800	Column	20	4.0	68	23	13	8	93
	4	3,600	Column → FACS	19	3.9	85	21	19	12	413+
	5	2,400	Column → FACS	15	3.2	91	20	24	21	184+
Depleted	6	1,300	Depletion	1,200	285.0	<1	177	126	112	227
	7	1,400	Depletion	920	184.0	<1	NR	NR	NR	29
Unseparated	8	950	None	950	206.0	ND	17	20	16	57
	9	1,200	None	1,200	218.0	ND	20	18	11	29+
No marrow	10	0	None	0	—	—	NR	NR	NR	17

Cell separation procedures and engraftment data are summarized for 10 animals. Five animals received $CD34^+$ enriched cells isolated by column immunoadsorption alone (baboons 1–3) or column immunoadsorption followed by flow microfluorimetric sorting (FACS, baboons 4 and 5). Baboons 6 and 7 received marrow depleted of $CD34^+$ cells by column immunoadsorption. Baboons 8 and 9 were controls and received unmodified whole marrow. Baboon 10 was given no marrow and served as radiation control. The table shows the day after transplant when each animal achieved neutrophil counts > 1,000/mm³, untransfused platelet count > 20,000/mm³, and the last day that animals required red cell transfusions. ND, not done; NR, not reached.

and marrow that showed trilineage engraftment at autopsy. One animal that was irradiated and given no marrow died at day 17 with marrow aplasia at autopsy (Table I).

Posttransplant syndrome and long-term survival. All animals surviving greater than 1 mo developed a syndrome characterized by atypical lymphocytosis, hemolytic anemia with elevated reticulocyte count, thrombocytopenia, and lymphadenopathy. During this illness, animals that were transplanted with $CD34^+$ cells had histologically normal marrows as determined by biopsy and in vitro assays demonstrated the presence of marrow colony-forming cells. A control animal given unprocessed marrow and the prolonged survivor transplanted with marrow depleted of $CD34^+$ cells also developed this syndrome. This syndrome had its onset between 1 and 4 mo after transplant. Five animals died with interstitial pneumonia and/or encephalitis during the illness, and two animals transplanted with $CD34^+$ cells recovered. One control animal, given unprocessed marrow, is < 1 mo posttransplant and will need longer follow-up to determine if it also develops this syndrome. The etiology of the illness has not been established although a viral agent is suspected. Bacteria and fungi have not been found nor have cytomegalovirus, Epstein-Barr virus, herpes viruses, or retroviruses been isolated from these animals.

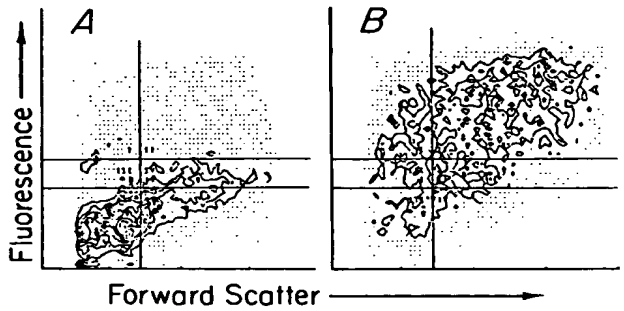

Figure 1. Marrow cells from second baboon before and after column separation. Unseparated marrow cells labeled with antibody 12-8 and biotinylated goat anti-mouse IgM antiserum (2A), and antibody-labeled adherent cells recovered from the column (2B) were stained with avidin-FITC and analyzed by flow microfluorimetry. The unseparated marrow cells were 1.5% $CD34^+$, while the recovered adherent cells were 81% $CD34^+$.

Discussion

Several monoclonal antibodies have been developed that identify the CD34 antigen expressed by early human hematopoietic progenitors detected by in vitro assays (3–6). One of these antibodies, 12-8, has the unique property of recognizing a distinct epitope that is also present on a similar marrow population in non-human primates (7). In the present study, highly enriched populations of $CD34^+$ cells isolated from baboon marrow were capable of completely restoring hematopoiesis when transplanted into lethally irradiated animals. The rate of engraftment in these animals was similar to that of control animals transplanted with unseparated marrow and was similar to that reported in previous studies of autografts in lethally irradiated nonhuman primates (15, 16). Two animals received $CD34^+$ cells that were further enriched by flow microfluorimetric sorting after immunoadsorption and demonstrated prompt engraftment and complete hematologic recovery. Therefore, the $CD34^-$ cells contaminating the adsorbed cell population are unlikely to have played a role in restoring hematopoiesis. The rapid and durable engraftment for more than 6 mo and 1 yr in these two animals suggests that the stem cells that produce hematopoietic reconstitution are contained

Table II. Colony-forming Cells Detected after Enrichment or Depletion of CD34⁺ Cells

	Baboon	Cell fraction	CFU-GM	BFU-E	CFU-MIX
				Colonies/10⁵ cells	
Enrichment	1	Unseparated	94±13	22±7	7±5
		Adherent	2500±280	500±80	200±44
		Nonadherent	40±6	9±3	2±1
	2	Unseparated	101±15	27±5	2±1
		Adherent	1356±101	197±53	123±29
		Nonadherent	37±5	13±2	1±0
	3	Unseparated	39±9	13±5	1±1
		Adherent	2246±346	1226±300	400±73
		Nonadherent	13±5	3±1	1±1
	4	Unseparated	81±12	60±9	7±4
		Adherent alone	1389±97	853±64	161±17
		Adherent → FACS CD34⁺	1966±217	1506±129	216±25
		Adherent → FACS CD34⁻	5±1	15±4	0±0
		Nonadherent	22±9	50±6	2±1
Depletion	6	Unseparated	80±7	40±5	5±1
		Adherent	1008±63	240±34	56±7
		Nonadherent	<1	2±1	<1
	7	Unseparated	56±9	36±3	4±4
		Adherent	309±34	118±10	25±6
		Nonadherent	4±1	15±2	0±0

Column immunoadsorption was used to either enrich (baboons 1–4) or deplete (baboons 6 and 7) CD34⁺ marrow cells. Marrow cells before column treatment (unseparated), cells bound to the column (adherent), and cells that passed through the column (nonadherent) were assayed for in vitro colony-forming progenitors (see Methods). In baboon 4, the adherent cells were further separated by flow microfluorimetric sorting (FACS) into CD34⁺ and CD34⁻ fractions. Data were not available for baboon 5 due to technical problems with the assays.

within the CD34⁺ cell population. Of two animals given marrow depleted of CD34⁺ cells, one died without evidence of engraftment, while the second animal demonstrated marrow aplasia for over 2 mo after transplant, pancytopenia for > 3 mo posttransplant, and continued to show marked hypoplasia of myeloid elements on serial marrow examinations until its death. The data suggest that CD34⁺ cells are both necessary and sufficient for complete hematopoietic reconstitution in vivo.

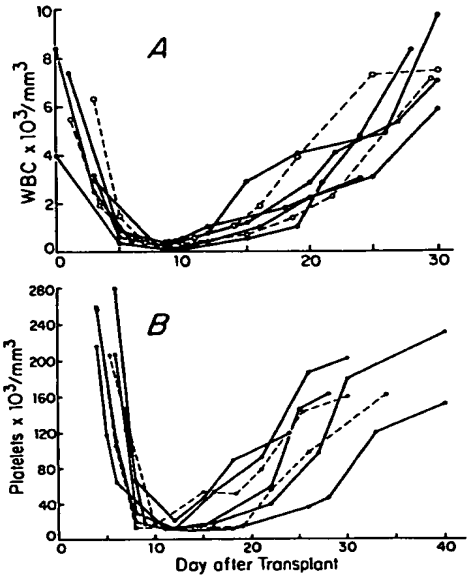

Figure 2. Total leukocyte count (2A), measured serially posttransplant in baboons transplanted with CD34⁺ cells (●) or whole marrow (○). Platelet count (2B) measured serially post-transplant in baboons transplanted with CD34⁺ cells (●) or whole marrow (○).

The possible contribution of endogenous radioresistant stem cells to hematopoietic reconstitution cannot be determined from these studies. The radiation dose used in these experiments resulted in lethal marrow aplasia in a control animal and has been shown previously to produce lethal marrow aplasia in baboons (17, 18). However, it is possible that transfused CD34⁺ cells supported early hematologic function allowing the animals to survive long enough for primitive progenitor cells to recover and sustain long-term hematopoiesis. The insertion of a genetic marker into the infused autologous cells or transplantation of allogeneic CD34⁺ cells will be required to prove the origin of cells responsible for hematopoiesis. Similarly, it cannot be determined whether partial hematologic recovery observed in one animal given marrow depleted of CD34⁺ cells was due to the recovery of stem cells in the irradiated recipient. It is also possible that inadequate depletion resulted in the infusion of a small number of CD34⁺ cells sufficient to allow the partial reconstitution of hematopoiesis. The development of more effective depletion methods and the use of genetic markers will be required to determine if either of these possibilities is correct.

A posttransplant syndrome of lymphadenopathy, atypical lymphocytosis, hemolytic anemia, and consumptive thrombocytopenia was observed in all transplanted animals surviving > 30 d. The illness has the characteristics of a viral disease,

but an etiology has not yet been identified. Animals receiving unmodified marrow, $CD34^+$ cells, or marrow depleted of $CD34^+$ cells have all developed the illness thus making it unlikely that the infusion of $CD34^+$ cells or the avidin-biotin immunoadsorption procedure per se caused this syndrome. Studies are in progress to identify the etiologic agent.

In humans and baboons, the $CD34^+$ marrow population is heterogeneous and contains immature B and T lymphocytes as well as myeloid progenitor cells (Andrews et al., manuscript submitted for publication; Andrews and Bernstein, unpublished observations; 3, 5, 19, 20). Furthermore, $CD34^+$ cells can be divided into functionally distinct progenitor populations based on expression of the CD33 antigen. In humans, the $CD33^-CD34^+$ progenitors are precursors of colony-forming cells in long-term marrow culture, while the $CD33^+CD34^+$ progenitors account for virtually all colony-forming cells (Andrews et al., manuscript submitted for publication). Studies to separate $CD34^+$ marrow cells into $CD33^-$, $CD33^+$, and lymphoid subpopulations will further define the nature and function of the progenitor cell populations required for reconstituting hematopoiesis in vivo.

The use of highly enriched populations of hematopoietic progenitor cells may have wide applicability to marrow transplantation. For example, if hematopoietic stem cells can be isolated without tumor cells, then this technique may provide an alternative approach to methods currently being used to deplete tumor cells ex vivo from the marrow of patients undergoing autologous marrow transplantation. Furthermore, positive selection could be used to isolate hematopoietic stem cells without T lymphocytes that cause graft-versus-host disease after allogeneic marrow transplantation. Finally, positive selection can provide a source of highly enriched hematopoietic progenitors for studies of gene transfer.

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